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# Regulation of $Ca^{2+}$-dependent protein turnover in skeletal muscle by thyroxine

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Dantrolene, an agent that inhibits $Ca^{2+}$ mobilization, improved protein balance in skeletal muscle, as thyroid status was increased, by altering rates of protein synthesis and degradation. Thyroxine ($T_4$) caused increases in protein degradation that were blocked by leupeptin, a proteinase inhibitor previously shown to inhibit $Ca^{2+}$-dependent non-lysosomal proteolysis in these muscles. In addition, $T_4$ abolished sensitivity to the lysosomotropic agent methylamine and the autophagy inhibitor 3-methyladenine, suggesting that $T_4$ inhibits autophagic/lysosomal proteolysis.

## INTRODUCTION

Thyroid hormones have profound effects on a variety of metabolic functions, including $O_2$ consumption, heat production, glycolysis, protein synthesis and proteolysis (Hoch, 1974; Goldberg *et al.*, 1977; van Hardeveld & Kassenaar, 1980; Brown & Millward, 1983; van Hardeveld & Clausen, 1984). Growth-promoting levels of thyroxine ($T_4$) stimulate both rates of protein synthesis and, to a lesser extent, proteolysis, whereas higher catabolic levels cause further stimulation of protein degradation (Brown & Millward, 1983; Goldberg *et al.*, 1977). Recently, it has been demonstrated that the muscle relaxant dantrolene blocks 3,3',5-tri-iodothyronine-('$T_3$')-stimulated $O_2$ consumption (van Hardeveld & Kassenaar, 1980). Since many studies have concluded that dantrolene is a specific inhibitor of $Ca^{2+}$ release from the sarcoplasmic reticulum (Desmedt & Hainaut, 1976; Francis, 1976; Taylor *et al.*, 1979), this result suggests that $Ca^{2+}$ may have a role in various $T_4$-altered metabolic processes.

The ability of $Ca^{2+}$ to alter rates of protein turnover of skeletal muscle (Kameyama & Etlinger, 1979; Rodemann *et al.*, 1980; Sugden, 1980; Lewis *et al.*, 1982; Silver & Etlinger, 1985; Zeman *et al.*, 1985, 1986) suggests a role for $Ca^{2+}$ in the $T_4$ regulation of protein balance as well. $T_4$-induced increases in levels of lysosomal proteinases in muscle had led to the suggestion that $T_4$ stimulates a lysosomal/autophagic pathway of protein degradation (DeMartino & Goldberg, 1978). However, tissue levels of proteinases may not necessarily reflect the activities of proteolytic pathways. Interestingly, the $Ca^{2+}$-stimulated increase in overall proteolysis in muscle is sensitive to leupeptin and E-64-c, but not to lysosomotropic agents nor the autophagy inhibitor 3-methyladenine, suggesting that a non-lysosomal proteolytic activity is stimulated by $Ca^{2+}$ (Zeman *et al.*, 1985).

We present evidence here suggesting that $T_4$ regulates overall protein turnover by enhancing $Ca^{2+}$-stimulated non-lysosomal protein degradation while inhibiting lysosomal/autophagic proteolysis by a $Ca^{2+}$-dependent mechanism. In addition, $Ca^{2+}$ stimulates rates of protein synthesis in hypothyroid muscle and $T_4$ enhances $Ca^{2+}$-dependent inhibition of protein synthesis caused by membrane depolarization.

## EXPERIMENTAL

### Materials

The sodium salt of $T_4$ was obtained from Sigma Chemical Co., as were phenylalanine, tyrosine, methylamine and cycloheximide. [$^{14}$C]Phenylalanine was purchased from New England Nuclear, dantrolene from Norwich–Eaton Pharmaceuticals (Norwich, NY, U.S.A.) and 3-methyladenine from Fluka. Leupeptin was a gift from Dr. Alfred Stracher, Downstate Medical Center, Brooklyn, NY, U.S.A.

### Animals

Hypophysectomized female Wistar rats from the Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) were injected daily, for 1–2 weeks before being killed, either with saline vehicle or with 2.5 $\mu$g or 200 $\mu$g of $T_4$. The low dose of $T_4$ increased the mean body weight from $63.0 \pm 0.8$ to $70.1 \pm 0.9$ g (mean $\pm$ S.E.M., $P < 0.0005$, $n = 119$) and the high dose decreased body weight to $57.5 \pm 1.3$ g (mean $\pm$ S.E.M., $P > 0.0005$, $n = 115$). Hypophysectomized rather than thyroidectomized rats were used in order to determine the effects of thyroid hormone on protein turnover independently of those of growth hormone (Goldberg *et al.*, 1977).

### Measurements of rates of protein synthesis and degradation

Freshly dissected paired soleus or extensor digitorum longus muscles were incubated in a shaker bath for 2 h at 37 °C in Krebs–Ringer bicarbonate solution (4 ml), gassed with $O_2/CO_2$ (19:1), containing 10 mM-glucose. Additions to the medium of [$^{14}$C]phenylalanine, 0.5 mM-phenylalanine (final specific radioactivity, 294 d.p.m./nmol), dantrolene (10 $\mu$g/ml), KCl (20 mM total $K^+$), leupeptin (10 $\mu$g/ml), 20 mM-methylamine and 10 mM-3-methyladenine were made before incubation. Since separate leupeptin- and lysosomotropic agent-sensitive components of proteolysis can be demonstrated in the absence of insulin and amino acids (Zeman *et al.*, 1985, 1986), they were not included in the medium.

Abbreviations used: $T_4$, (L-)thyroxine (3,3',5,5'-tetraiodothyronine); $[K^+]_o$, extracellular $K^+$ concentration; EDL, extensor digitorum longus.

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**Table 1. Effects of dantrolene and $K^+$ on protein synthesis (*a*) and protein degradation (*b*) in muscle of increasing thyroid status**

Rates of protein synthesis and degradation of soleus muscles were measured simultaneously during 2 h incubations under the conditions described in the Materials and methods section. The muscles were obtained from hypophysectomized rats injected daily, for 1–2 weeks before they were killed, with either saline vehicle or 2.5 $\mu$g of $T_4$/day, as indicated. The values presented are the means ± S.E.M. for paired control and dantrolene-treated (10 $\mu$g/ml) muscles. The statistical significance of the effects of adding dantrolene to the medium were determined with the Student's *t* test (two-tailed) for paired differences ($n$ = 5–8 observations). Significance: * $P < 0.05$; ** $P < 0.02$, *** $P < 0.001$; n.s., not significant.

| | Protein synthesis (nmol of tyrosine/h per mg) | |
|---|---|---|
| (*a*) Addition | Saline | $T_4$ (2.5 $\mu$g/day) |
| None | 0.041 ± 0.003 | 0.043 ± 0.002 |
| Dantrolene | 0.035 ± 0.002 | 0.047 ± 0.003 |
| Change (%)... | −15* | +9 n.s. |
| 20 mM-$K^+$ | 0.029 ± 0.002 | 0.021 ± 0.002 |
| 20 mM-$K^+$ + dantrolene | 0.043 ± 0.003 | 0.040 ± 0.005 |
| Change (%)... | +48** | +91*** |

| | Protein degradation (nmol of tyrosine/h per mg) | |
|---|---|---|
| (*b*) Addition | Saline | $T_4$ (2.5 $\mu$g/day) |
| None | 0.151 ± 0.011 | 0.183 ± 0.009 |
| Dantrolene | 0.159 ± 0.003 | 0.185 ± 0.010 |
| Change (%)... | +5 n.s. | +1 n.s. |
| 20 mM-$K^+$ | 0.182 ± 0.008 | 0.211 ± 0.005 |
| 20 mM-$K^+$ + dantrolene | 0.184 ± 0.010 | 0.188 ± 0.010 |
| Change (%)... | +1 n.s. | −11* |

Rates of protein synthesis and degradation were determined simultaneously using the incorporation of [$^{14}$C]phenylalanine into muscle protein and the release of tyrosine into the muscle pools and the medium (Tischler *et al.*, 1982). Since the ratio of tyrosine to phenylalanine content of protein in rat skeletal muscle is 0.77 (Tischler *et al.*, 1982), this factor was used to calculate rates of tyrosine incorporation. In some experiments, protein degradation was measured in the presence of 0.5 mM-cycloheximide and the absence of phenylalanine. All values are expressed relative to initial muscle wet weights. Tissue swelling was minimal during incubation, since measurements of final wet weights showed gains of 3–10%. As noted previously (Kameyama & Etlinger, 1979), rates of protein synthesis and degradation exhibited day-to-day variation. For this reason the degree of significance of values of percentage change were calculated for paired differences using the Student's *t* test ($n$ = 5–10).

## RESULTS AND DISCUSSION

To determine whether $Ca^{2+}$ has a role in the regulation of muscle protein turnover by thyroid hormone, the effect of dantrolene on rates of overall protein synthesis and degradation was measured. Dantrolene produced a small reduction (15%) in protein synthesis in muscles incubated in 6 mM-$[K^+]_0$ from hypothyroid but not $T_4$-treated rats (Table 1). This suggests that $Ca^{2+}$ can stimulate rates of protein synthesis under conditions of thyroid-hormone deficiency.

In contrast, dantrolene stimulated protein synthesis in muscle incubated in depolarizing medium containing 20 mM-$[K^+]_0$, from both groups of rats (Table 1). The stimulation was greater (40 as against 91%) in the $T_4$-treated group compared with saline-injected controls. However, a comparison of muscles incubated without dantrolene shows that elevated $[K^+]_0$ itself decreases protein synthesis and that this effect is also greater in the $T_4$-treated group (27 as against 51%). Thus the overall effect of dantrolene was to antagonize the inhibition of protein synthesis caused by membrane depolarization. The effect of depolarization on protein synthesis and its reversal by dantrolene are consistent with an ability of $Ca^{2+}$ to inhibit protein synthesis, as demonstrated in other studies with the agents (caffeine, thymol and procaine) that also mobilize $Ca^{2+}$ and decrease synthesis (Lewis *et al.*, 1982). The observations presented here suggest that protein synthesis is stimulated by low levels of $Ca^{2+}$ and inhibited by higher $Ca^{2+}$ levels produced by depolarization. The larger reduction in protein synthesis caused by depolarization in $T_4$-treated muscles as compared with hypothyroid muscle is consistent with an enhancement of $Ca^{2+}$ mobilization caused by thyroxine (van Hardeveld & Kassenaar, 1980; van Hardeveld & Clausen, 1984). Our observation that protein synthesis is both $T_4$- and membrane-potential-dependent may be related to findings that regulation of contractile-protein content and isoform distribution by $T_4$ is dependent on innervation (Johnson *et al.*, 1980).

Although replacement of $T_4$ at levels used here was previously shown to increase rates of protein synthesis (Goldberg *et al.*, 1977), no differences in the mean values are apparent in the present experiments. However, we were only concerned with paired differences assayed at the same time in the present studies. Changes in rates of protein synthesis caused by thyroid hormone may have been obscured by the greater variability inherent in

**Table 2. Effects of leupeptin, methylamine and 3-methyladenine on proteolysis in muscle of increasing thyroid status**

Rates of protein degradation of (*a*) soleus and (*b*) EDL muscles were measured in the presence of 0.5 mM-cycloheximide, during 2 h incubations under the conditions described in the Materials and methods section. The muscles were obtained from hypophysectomized rats injected daily for 1–2 weeks with either saline vehicle or $T_4$ as indicated. The values presented are the means ± S.E.M. for paired control and inhibitor-treated muscles. The statistical significance of the effects of adding the indicated inhibitors to the medium were determined with the Student's *t* test (two-tailed) for paired differences ($n = 5$–10 observations). Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$; n.s., not significant.

| Addition | Protein degradation (nmol of tyrosine/h per mg) | | |
|---|---|---|---|
| | Saline | $T_4$ (μg/day)... 2.5 | 200 |
| (*a*) Soleus | | | |
| None | 0.231 ± 0.005 | 0.295 ± 0.017 | 0.306 ± 0.021 |
| Leupeptin | 0.206 ± 0.015 | 0.193 ± 0.013 | 0.169 ± 0.031 |
| Change (%)... | −11* | −35** | −45*** |
| None | 0.205 ± 0.007 | 0.248 ± 0.033 | 0.281 ± 0.017 |
| Methylamine | 0.128 ± 0.006 | 0.238 ± 0.042 | 0.260 ± 0.054 |
| Change (%)... | −38**** | −4n.s. | −8n.s. |
| (*b*) EDL | | | |
| None | 0.145 ± 0.005 | 0.192 ± 0.011 | 0.264 ± 0.009 |
| Leupeptin | 0.128 ± 0.007 | 0.106 ± 0.020 | 0.134 ± 0.006 |
| Change (%)... | −12n.s. | −45*** | −49**** |
| None | 0.146 ± 0.008 | 0.162 ± 0.011 | 0.281 ± 0.017 |
| 3-Methyladenine | 0.110 ± 0.010 | 0.159 ± 0.014 | 0.272 ± 0.030 |
| Change (%)... | −25*** | −2n.s. | −3n.s. |

unpaired experiments performed on different groups of animals.

A concomitant finding with these muscles was that dantrolene also reduced protein degradation by 11%, but only in $T_4$-treated depolarized muscles (Table 1). This small, but significant, effect was similar to our previous studies showing that dantrolene reduces proteolysis in depolarized muscle from normal rats (Zeman *et al.*, 1985). The absence of an effect of dantrolene on protein degradation in muscles from hypothyroid rats (Table 1) suggests that thyroxine stimulates proteolysis by a $Ca^{2+}$-dependent mechanism. Although dantrolene affects both rates of protein synthesis and and degradation, these effects occur under different conditions of thyroid status and membrane potential. Since it is known that protein synthesis increases with increased thyroid status, a consistent hypothesis is that low levels of $Ca^{2+}$ mobilized by thyroid hormones stimulate protein synthesis, whereas larger extents of $Ca^{2+}$ movement caused by depolarization and potentiated by $T_4$ inhibit synthesis and enhance proteolysis.

To characterize $T_4$-stimulated protein degradation, we used the proteinase inhibitor leupeptin (Toyo-Oka *et al.*, 1978), the lysosomotropic agent methylamine (Seglen *et al.*, 1979) and the autophagy inhibitor 3-methyladenine (Seglen & Gordon, 1982). Previous studies showed that leupeptin inhibits $Ca^{2+}$-stimulated proteolysis that appears in $Ca^{2+}$-ionophore-treated muscle or on depolarization (Rodemann *et al.*, 1980; Zeman *et al.*, 1985). Recent studies support the conclusion that such $Ca^{2+}$-stimulated proteolysis is non-lysosomal, since such enhanced protein degradation occurs in the presence of a large variety of agents that inhibit the lysosomal/autophagic pathway of protein degradation in muscle as well as other cell types (Zeman *et al.*, 1985, 1986). In addition, in muscles treated with leupeptin, the $Ca^{2+}$ ionophore decreases rather than increases proteolysis (Zeman *et al.*, 1986). The pathway that is inhibited by $Ca^{2+}$ appears to be lysosomal, since the ability of several lysosomal inhibitors to decrease protein degradation is blocked in the presence of $Ca^{2+}$ ionophore. Thus these inhibitors can be used to distinguish between lysosomal and non-lysosomal pathways of protein degradation that are regulated by $Ca^{2+}$. The inability of leupeptin to inhibit lysosomal proteolysis in adult skeletal muscle (Zeman *et al.*, 1986) as opposed to other cell types (Seglen *et al.*, 1979; Silver & Etlinger, 1985) is unexplained, but may be due to some difference in access to cellular compartments.

Although leupeptin produced little or no effect on protein degradation in muscles from saline-injected rats, it decreased proteolysis in hormone-treated muscles in a $T_4$-dose-dependent manner (Table 2). The overall effect of leupeptin was to inhibit the increases in protein degradation simulated by $T_4$. In contrast, methylamine or 3-methyladenine acted oppositely, since proteolysis was decreased only in the saline-injected group (Table 2). The effects of leupeptin on protein degradation in either fast extensor digitorum longus (EDL) muscles or slow solei were indistinguishable. In addition, 3-methyladenine did not affect the proteolysis of solei from rats treated with the high dose of $T_4$ (200 μg/day; results not shown). Likewise, methylamine treatment produced no effect in EDLs of similar thyroid status (results not shown). This pattern of effects was similar to that observed with $Ca^{2+}$ ionophore-treated muscles, in which leupeptin-sensitive proteolysis is enhanced and sensitivity to lysosomal inhibitors is reduced (Zeman *et al.*, 1986). Thus $T_4$ seems to increase $Ca^{2+}$-dependent non-lysosomal proteolysis and to inhibit $Ca^{2+}$-regulated lysosomal/autophagic protein degradation.

An attractive possibility is that increased activity of $Ca^{2+}$-activated neutral proteinase, which is known to be leupeptin-sensitive and to degrade myofibrillar proteins, accounts for $T_4$-stimulated protein degradation (Toyo-Oka *et al.*, 1978). The species of this enzyme, calpain I,

that requires only micromolar concentrations of $Ca^{2+}$ for activation has recently been shown to be located throughout the sarcoplasm and does not appear to be associated with lysosomes (Kleese & Goll, 1984; Goll *et al.*, 1985). In addition, $T_4$ stimulates calpain activity in skeletal, but not in cardiac muscle, where increased thyroid status does not stimulate overall protein degradation (Toyo-Oka, 1980; Crie *et al.*, 1983). Since thyroxine also stimulates the activities of the lysosomal cathepsins B and D in lysosomal homogenate fractions (DeMartino & Goldberg, 1978; Toyo-Oka, 1980), it has been suggested that $T_4$ stimulates the lysosomal/autophagic pathway of proteolysis (DeMartino & Goldberg, 1978). Decker & Wildenthal (1981), using immunohistochemical techniques, have observed increased paranuclear localization of extralysosomal cathepsin D in hyperthyroid skeletal muscle. Although there was no evidence that the presence of the enzyme altered muscle ultrastructure, it is possible that cathepsin D or other lysosomal proteinases, which may have some activity at cytosolic pH levels, could enhance overall proteolysis. However, in view of our observations with inhibitors, conclusions based only on enzyme activities of muscle homogenates seem unwarranted, especially since there is no evidence that proteinase levels limit overall rates of proteolysis.

Studies by Dice & Walker (1978, 1980) on the selectivity of protein degradation under different conditions where overall proteolysis is enhanced, suggest that hyperthyroidism involves selective regulation of degradative pathways which differ from that seen under other conditions where overall proteolysis is enhanced. For example, correlations between protein half-life, charge, size and glycosylation are lost or markedly decreased when overall rates of proteolysis increase with insulin deficiency, while enhanced proteolysis associated with increased $T_4$ results in a loss of only the correlation with charge. These results imply that different pathways of protein degradation are stimulated in these two catabolic situations. Our evidence suggests that non-lysosomal leupeptin-sensitive proteinases may be responsible for muscle wasting in thyrotoxicosis or in other conditions where $Ca^{2+}$ may be elevated, e.g. certain hereditary dystrophies (Kameyama & Etlinger, 1979). Thus dantrolene, or proteinase inhibitors like leupeptin, may have potential therapeutic importance under these conditions.

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